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14. ABSTRACT Our objectives/hypotheses are obtaining additional diagnostic and prognostic insight through the analysis of tumor-related DNA integrity and methylation in prostate cancer patient sera, we are developing optimized, robust, and reproducible blood assays. In the past grant period we have been accruing patients' blood specimens. Currently we are optimizing assays and final validation. have moved forward to validate our initial findings on cell lines and tumor specimens in serum and plasma. The Alu qRT assay is being investigated using plasma. In a study of 57 prostate patients, we found significant differences of unmethylated LINE1 (uLINE1) status in patients compared to normal healthy male. The data is in final stage of manuscript preparation for manuscript submission. Assays for the biomarkers will be further carried out for the direct quantitative RealTime PCR (qRT) Alu and direct qRT LINE1 is being optimized. We will also continue to develop circulating DNA methylated GSTP1 assay to complement the DNA integrity biomarkers (Alu and LINE1). The final validation of the assay specimens will be completed and manuscript(s) will be submitted.					
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INTRODUCTION

Proposed work has been delayed in this period due to personnel change and assay optimization and validation issues. Patient accrual has been continued. We have requested a no cost extension to complete the various tasks. The manuscript on the uLINE1 work so far described is in its final stage of preparation for submission. We are currently working on validating the uLINE1 assay in another independent group of PCa patients and normals. This is important to demonstrate the robustness and reproducibility of this promising blood assay. We've had to overcome challenges of continuing this assay after one of the researchers left the project. As for the direct qRT assay to assess for free circulating LINE1 DNA, we have an ongoing assay assessing DNA extracted from serum. We are moving forward in establishing the detection directly in serum without DNA extraction. The Alu direct qRT assay is being investigated comparing serum and plasma for clinical utility and assay feasibility. The LINE1 assay took precedent in that it developed faster and showed quality reproducible results. We have a defined set of prostate and benign patient specimens procured so far reaching our proposed number of specific types of patients relative to disease status to assess in this project. We are actively following up on the clinical and demographical data to verify that all specimens will be useful toward our objective/hypothesis of obtaining additional diagnostic and prognostic insight through the analysis of tumor-related DNA integrity and methylation in patient serum. The time delay in part was to accrue patients of specific disease status and follow up to have meaningful data.

In this program year, we have had some significant progress on the assay development. It has been difficult in establishing assays to obtain robust and reproducible results. We are finding multiple confounding factors influencing the circulating DNA assays. We have however developed the LINE1 assay, assembled the manuscript on uLINE1, and performed preliminary analysis of circulating DNA GSTP1 methylation. The goal is to validate the clinical utility of the assays and biomarkers.

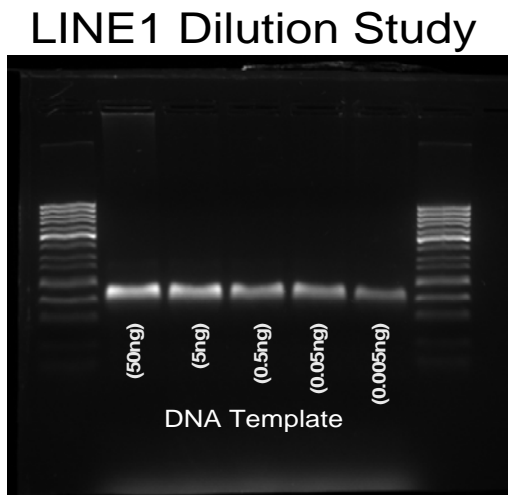
BODY

In this year of the proposal period, we continue to accrue prostate cancer (PCa) patients and normal donor serum for the study. We currently have serum specimens from over 200 PCa patients and over 80 normal donor serum collected. We also have reached the 25 prostatitis patients, but we are struggling to enroll patients with benign prostate hyperplasia conditions. Some of these samples have been used in the optimization and pilot studies of the various assays proposed.

We continue to develop Alu assay for interrogating serum samples. There are potentially two testing sites for the circulating Alu, the plasma and serum. Direct quantitative Realtime PCR (qRT) assay may be further optimized in the plasma since it does not contain fibrinogen or the other clotting factors. The direct assay does not require DNA extraction before qRT. This avoids problems with low DNA yields when extracting from serum or potential false positives. We are exploring the option of assessing plasma and determining clinical utility of the assay in plasma.

Direct qRT for circulating LINE1 in serum or plasma has been developed. Low levels of LINE1 can be detected in normal age-matched male donors. A dilution of cell line DNA has demonstrated sensitivity from 50ng to 0.005ng of DNA (**Figure 1**). From 500ul of serum, total of 50-500ng of DNA can be extracted. This is a good yield from our procedure we have developed. We are working on quantifying the level of LINE1 detection and further optimizing the assay to detect LINE1 in as low as 50 ul of serum directly. This would allow multiple assays to be performed with limited amount of serum. We plan to move forward with this assay once our pilot studies of PCa patient sera show clear discriminatory level of LINE1 detection from normal and benign prostate patients.

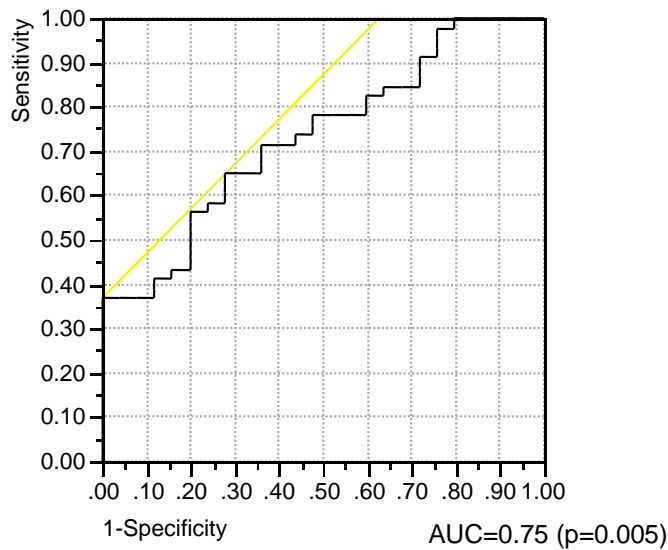
Figure 1



As reported in our last progress report, we have developed the uLINE1 AQAMA (absolute quantitative allele methylation assay) for the assessment

of PCa patient serum. The results of the study of 57 PCa patients have been assembled for manuscript submission. The study investigated several ways of using LINE1 as biomarker for prostate cancer. For the utilization of LINE1 hypomethylation, first we applied LINE1 hypomethylation index, which indicates the ratio of unmethylated LINE1 / total (unmethylated and methylated) LINE1. PCa patients showed 1.4 times higher LINE1 hypomethylation index and AUC value for this assay reached 0.75 (**Figure 2; ROC curve**). Because we use the ratio for this assay, it is not necessary to be attentive to the amount of DNA derived from fixed volume of serum. There are several steps to perform AQAMA assay for circulating DNA such as DNA extraction from serum, sodium bisulfite modification (SBM) treatment and purification of DNA.

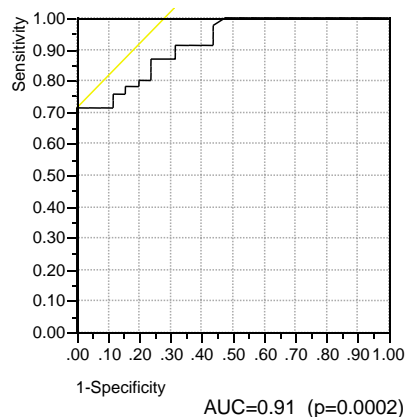
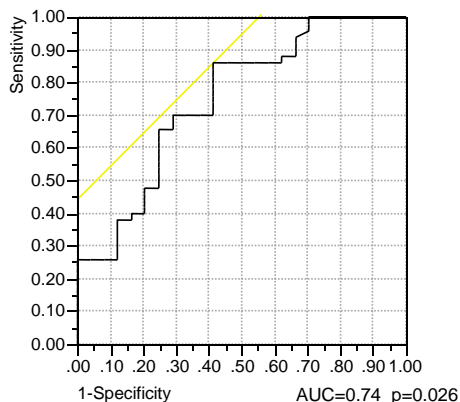
Figure 2



Second quantification of LINE1 was investigated as to whether the assay can be used for screening. Serum of PCa patients contains about 2 to 3 times higher amount of circulating LINE1 DNA and ROC AUC value for this analysis was 0.74 (**Figure 3; ROC curve**). This is an assay system performed by means of quantitative realtime PCR without methylation analysis, so it is easier than other assays. Finally unmethylated LINE1 copy number was tested regarding capability for screening use. It represents combination of both the hypomethylation index and DNA quantification. The ROC AUC value for this analysis was 0.91 (**Figure 4; ROC curve**) and this result indicates that unmethylated LINE1 copy number in serum circulating DNA can be applied for PCa screening.

Figure 3

Figure 4



LINE1 hypomethylation index, quantification of LINE1 and quantification of unmethylated LINE1 showed no differences between PSA normal and high groups, which means those three biomarkers are independent of PSA. From those features, the combination use of those biomarkers and PSA may improve the screening ability of those three assays. Among 13 PSA normal patients with cancer, seven patients showed higher copy number of unmethylated LINE1. In other words, we can detect cancer in about half of the PSA normal PCa patients using uLINE1.

In conclusion hypomethylation of LINE1 was recognized in serum circulating DNA drawn from PCa patients than those from healthy males. The ROC curve of unmethylated LINE1 quantification of serum DNA for discriminating patients with PCa (n=57) from healthy males (n=25) showed an area under curve (AUC) value of 0.91 (p=0.0002). When the specificity was set to 80%, sensitivity for PCa prediction was 81% and the cut-off value of unmethylated LINE1 copy number was about 4.0×10^3 . This minimally invasive assay targeting uLINE1 in circulating DNA is promising approach as a screening tool for PCa. The most important finding is that PCa patients with low PSA can be detected with circulating DNA biomarker. This is highly important in screening and monitoring PCa patients. A major problem in PCa diagnosis using a blood test is the high false positive rates of PSA. The combination of PSA and circulating DNA may be of very significance importance. This coming period we will focus on these aspects of the assay program.

KEY RESEARCH ACCOMPLISHMENTS

- Task 1 is 85% completed with >200 PCa patient, 25 prostatitis, and >80 normal healthy male donor serum collected. We are currently working on QC of the specimens and clinical data collection.
- Task 2 is in progress with optimization of Alu direct qRT for serum near completion.
- Task 3 optimization of circulating LINE1 assessment is completed in serum and we are currently evaluating the use of plasma to interrogate LINE1.
- Task 4 is semi-completed with patient and normal donor serum samples. We are working on assembling the data for publication. A second set of specimens will be evaluated to validate the uLINE1 assay.
- Task 5 the accrual of patients has been slow and we are still hoping we can reach its accrual. Alternative sources are being looked at for patient accrual.
- Task 6 is in progress with data analysis completed for the manuscript and patient sample size determination completed for assay efficiency investigation.

REPORTABLE OUTCOMES

No reportable outcomes have occurred during this time period. A manuscript will be submitted this year.

CONCLUSIONS

Although some progress has been made this year due to assay optimization/validation difficulties, we are pushing forward to validate the established assay and develop new assays. We are unable to get enough patients enrolled to generate meaningful data for Task 5 at this point. We will focus more on completing other tasks during the current year.

REFERENCES

None.

APPENDICES